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A Simplified Method for Virus-Tissue Culture Procedures in Microtitration Plates.* (28325)

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The method reported herein is a modification of the Takatsy(1) serological microtitration technique adapted to conventional tissue culture procedures, and recently described by Sever(2). Briefly, the titration plate (Fig. 1) simulates a series of micro tissue culture tubes in which cell monolayers are grown on the hemispherical bottom of the well compartment. Rapid serial dilution of virus or serum can be accurately accomplished by loop transfer.

Materials and methods. Microtitration apparatus was obtained from a commercial source† and consisted of Plexiglas plates moulded to contain 96 cups, each of which has a working capacity of approximately 0.2 ml; wire spiral loops with a calibrated transfer of 0.025 ml; and micropipets which deliver 0.025 ml/drop (Fig. 1).

Plates and micropipets were soaked in detergent and washed in tap water, followed by 3 rinses in triple distilled water. Virus-contaminated plasticware was soaked in a solution of 0.5% sodium hypochlorite and then washed as above. The utensils were then air-dried before further treatment.

Plates were irradiated with a 30W germici-

* The opinions and assertations contained herein are those of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

† Cooke Engineering Co., Alexandria, Va.

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dal lamp for 1 hour,‡ and micropipets were autoclaved before use. Wire diluting loops were flame-cleaned and sterilized before serum or virus dilutions were carried out. The operation of the test was carried out under "open" conditions.

Diluent consisted of 0.5% lactalbumin-hydrolysate in Hanks' balanced salt solution (HBSS). Tissue culture growth medium contained 90% Eagle's minimum essential medium (MEM) in HBSS with 10% inactivated fetal calf serum. Although only growth media was used for microtitration tissue cultures (MTC), conventional (MACRO) tissue cultures in tubes were changed to maintenance medium (95% MEM and 5% inactivated horse serum) once monolayers were established. All media contained penicillin (200 units/ml), streptomycin (200 μg/ml), and Amphotericin B§ (5 μg/ml).

Serum samples for the poliovirus neutralization test were obtained from 151 naval recruits. Twenty-eight of these individuals were then vaccinated with commercial live oral Type III poliovirus vaccine and second serum samples were obtained 42 days later. All specimens were inactivated by heat (56°C/30 min) prior to dilution. Poliovirus Type III was obtained from the American Type Culture Collection, Washington, D. C. and a large pool was made in H.Ep-2 monolayers for both the MTC and MACRO tests.

H.Ep-2 cell suspensions (3-5 $\times$ 10^5 cells/ml) were prepared from either trypsin-versene dispersed monolayers or spinner cultures.

MTC procedures. Poliovirus infectivity titer was determined by making serial $\log_{10}$ dilutions of pooled stock virus in diluent in 11 $\times$ 25 mm metal capped tubes. Eight replicates of each virus dilution were made by loop transfer to plate cups containing 0.025 ml of diluent (simulated serum dilution).‖ H.Ep-2 cells were added to virus dilutions by micropipet 1 drop (0.025 ml) per cup (7,500-12,500 cells). Included in each plate were 8 cup cultures which did not contain virus and served as cell controls. All cups were finally overlaid with 1 drop of mineral oil¶ (0.05 ml) to prevent dehydration and prolonged alkalinity. The total volume of the ingredients of each cup was 0.125 ml.

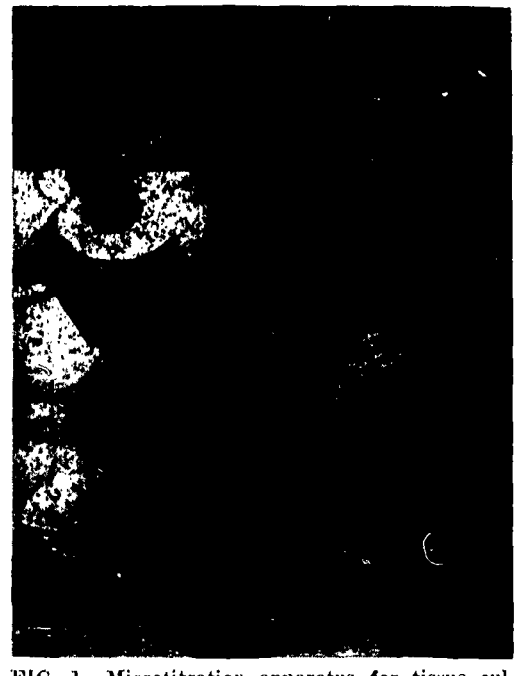

FIG. 1. Microtitration apparatus for tissue culture techniques. Foreground: Inverted microscope with microplate on the microscope stage. Background: Cell suspension on a magnetic stirrer; micropipets and loops in racks.

Plates were incubated in a humidified bacteriological incubator (35°C $\pm$ 1°) in a stacked manner which provided cover lids for the underlying plates and also conserved space.

Control tissue culture cell compartments showed confluent outgrowth after 48 hours of incubation and virus infectivity titers were microscopically observed and recorded at this time.

‡ Recent information indicates that prolonged exposure of Plexiglas plates to ultra-violet light will induce structural weaknesses and result in plate damage.

§ Fungizone—E. R. Squibb & Sons, New York.

‖ Once the approximate virus infectivity titer is known, it can be verified by serial 2-fold dilutions in the plates in a dilution range which brackets the actual titer.

¶ Drakeol—Pennsylvania Refining Co., Butler, Pa.

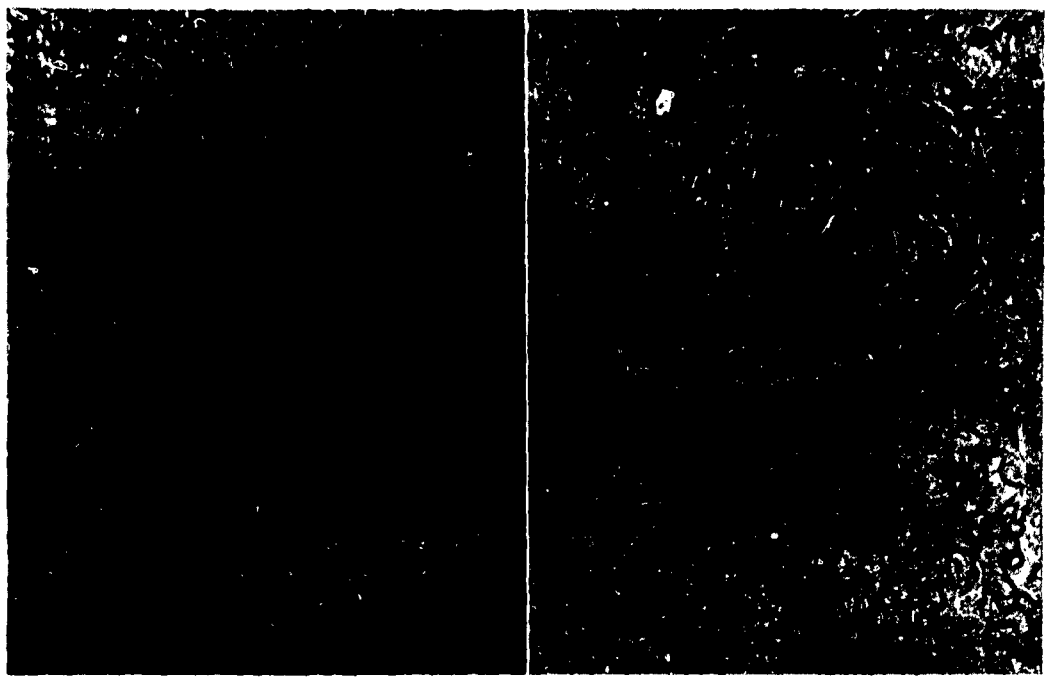

FIG. 2. Microscopic appearance of unstained H.Ep-2 microplate monolayers. Normal cells (left) and poliovirus Type III infected cells (right). (99×)

Microplate cell cultures were observed with the aid of the Leitz inverted microscope** (Fig. 1) which facilitates microscopic viewing of the plates without loss of the cup contents. The microscopic appearance of normal and poliovirus infected MTC monolayers is shown, respectively, in Fig. 2.

Infectivity titers were made on the basis of conventional 1+ - 4+ (25-100%) evidence of cytopathic effect (CPE). Fifty percent or greater CPE was indicative of infection. Infectivity titers were calculated by the method of Kärber(3).

Dilutions of serum antibody for neutralization tests were accomplished in the plate cups containing diluent with the aid of the transfer loop. Initial serum was diluted 1:20 in quadruplicate cups with one pair serving as the serum toxicity controls. Virus dosage (500 TCD_{50}/.025 ml) was delivered by micropipet (1 drop/cup) to duplicate serum dilutions and the mixtures were incubated at room temperature for 30 minutes. Subsequent addition of cells, oil, incubation and titration assay were carried out as described above. Inhibition of 2 + CPE was considered as protective.

MACRO procedures. Tissue culture tubes were prepared by inoculation of 60,000 H.Ep-2 cells contained in one ml of growth media per tube. These culture tubes were incubated at 35°C for 48 hours at which time islands of cells were established (the time required to complete the MTC test). At this time the culture fluids were removed and replenished with maintenance media.

Virus infectivity dilutions were carried out as in the MTC test and 0.1 ml of dilution was inoculated into quadruplicate culture tubes.

Inoculated tubes were reincubated for another 48 hours at 35°C and read for microscopic evidence of CPE. Titer endpoints were evaluated in a manner similar to that described in the MTC procedure.

Neutralization tests were performed by addition of equal volumes of 500 TCD_{50} to 2-fold serum dilution in a separate tube and mixtures were incubated at room temperature for 30 minutes. Two-tenths ml of the mix-

** Leitz microscope—supplied by W. H. Kessel & Co., Scientific Instruments, Chicago, Ill.

TABLE I. Comparison of Neutralizing Antibody Titers* to Poliovirus Type III Determined by Microtitration (MTC) and Tissue Culture Tube Methods.

Neutralizing antibody titers (reciprocal of initial dilution)							
Tissue culture tube (MACRO)							
MTC	>320	320	160	80	40	20	<20
>320							1
320				2	1	-	1
160			3	2	-	1	
80		2	1	-	1		
40	3	4	2	3			
20	10	4	11	2			
<20	77	15	4	1			
	<20	20	40	80	160	320	>320

* Serum samples from 151 naval recruits.

ture was added to duplicate tissue culture tubes containing established monolayers and antibody titer was assayed as described above.

Results. Infectivity titers of Type III poliovirus as measured by the MACRO and MTC technics were $10^{7.0}$ and $10^{7.1}$ respectively.

A comparison of poliovirus neutralizing antibody tests performed by both methods on 151 single sera (Table I) indicates good agreement of antibody titers was obtained. However, since titer endpoints were not determined on 78 sera (77 with titers $<$ 1:20; 1 with titer $>$ 1:320), a complete analysis could not be made. Therefore, the 28 paired serum specimens were titrated again by both methods (MTC and MACRO) at dilutions extending from 1:2 to 1:2048. The results of these 56 neutralization tests are shown in Table II. It can be seen that by MTC retitration of the 28 prevaccination sera, one (3.6%) failed to reproduce antibody titers less than the 1:20 dilution; whereas 3 of the 28 sera remeasured by the MACRO test (10.7%) showed titers in excess of the 1:20. The ratio of significant serological conversions noted in Table II by the MTC test was 21/28 (75%) as compared to 18/28 (64%) in the MACRO test. However, the number of agreements in both tests as to serologic conversion or not was 25/28 (89.3%).

In these 56 neutralization tests (pre and post vaccination sera) the number of sera whose titer did not vary more than 2-fold by either test was 45 (80.3%), indicating substantial agreement of the two techniques.

Bacterial or fungal contamination occurred in less than 0.1% of the total number of microplate wells employed.

Discussion. Virus titrations and serum neutralization tests with either suspensions of cells or established monolayers in plastic plates have been used by various investigators for enterovirus(2,4-6) adenovirus(7,8) and influenza virus(9). These investigators assayed virus and antibody titers by the colorimetric or metabolic inhibition procedures.

Although the colorimetric estimation technique can be of advantage where clear-cut consistent indicator changes occur due to viral infection, many virus-host cell systems do not produce such effect. Lennette(7) observed that indicator changes were affected by the cell concentration, and that accurate enumeration of cells was essential to formation of significant color changes. In most of the past studies, estimates of antibody titer by color were 3- to 4-fold higher than by microscopic observation. This discrepancy was also observed in our MTC study.

Therefore, it is believed that evaluation of CPE remains the best criterion for the presence and quantitation of virus. The methods described here show how this evaluation can be easily and accurately attained.

The requirements for reagents, time of preparation and operation are considerably reduced from those of the disposable plate and greatly reduced from tissue culture tube methods.

Microscopic viewing of cell monolayers is facilitated since 96 closely spaced wells (equivalent to approximately one and one-half commercial tissue culture tube racks) are incorporated on a single plate reducing the number of manipulations. An entire cell monolayer can be observed in 1 to 4 microscopic fields (depending on the microscope

TABLE II. Comparison of Poliovirus Type III Neutralizing Antibody Titers and Serological Conversions in Paired Human Sera by Microtitration Plate (MTC) and Tissue Culture Tube (MACRO) Methods.

Paired sera	Neutralizing antibody titers by test method (reciprocal of dilution)							
	MTC				MACRO			
	Prevaccination titer Determination (1)*	(2)	Post vaccination titer	4-fold or > titer increase	Prevaccination titer Determination (1)*	(2)	Post vaccination titer	4-fold or > titer increase
1	<20	<2	4	+	<20	<2	8	+
4	<20	<2	64	+	<20	<2	64	+
9	<20	2	128	+	<20	4	128	+
17	<20	8	128	+	<20	16	128	+
20	<20	4	1024	+	<20	8	1024	+
21	<20	16	64	+	<20	32	16	—
32	<20	16	128	+	<20	32	128	+
36	<20	4	32	+	<20	8	32	+
39	<20	2	32	+	<20	2	16	+
45	<20	2	128	+	<20	2	64	+
47	<20	2	8	+	<20	2	8	+
48	<20	2	1024	+	<20	2	1024	+
50	<20	2	128	+	<20	2	32	+
55	<20	<2	32	+	<20	<2	64	+
70	<20	2	2	—	<20	2	4	—
82	<20	<2	16	+	<20	<2	4	+
87	<20	16	256	+	<20	8	16	—
97	<20	2	2	—	<20	8	8	—
102	<20	4	64	+	<20	8	64	+
108	<20	16	32	—	<20	16	32	—
112	<20	16	128	+	<20	8	16	—
117	<20	32	256	+	<20	16	256	+
119	<20	16	32	—	40	64	32	—
122	<20	2	16	+	<20	2	8	+
123	<20	4	128	+	<20	8	64	+
142	<20	16	16	—	40	64	32	—
146	<20	4	8	—	<20	8	16	—
150	<20	8	16	—	<20	32	16	—
Total 28				21 (75%)				18 (64%)
Geometric mean titer		4.3	45.0			6.1	34.0	

* Data from Table I.

objective used); whereas many more field-shifts are required when appraising a tissue culture tube. Not only does the MTC procedure lessen the burden of decision upon the viewer in evaluating extent of CPE, but greater replication of titrations can be easily made to increase statistical reliance.

The discrepancies in antibody titer end-points observed between the MTC and MACRO tests may be due to the inherent error in the reproducibility of either test.

It should be noted that in the 28 sera re-run by both tests, a smaller error (3.6%) was observed in the MTC than in the MACRO test (10.7%). This error may also be involved in the 11% differences noted in the number of serological conversions produced by poliovirus Type III vaccination. Further studies should be done comparing antibody titers produced by both techniques with a third parameter such as plaque reduction methods to determine which of the two former most accurately measures virus antibody.

Nevertheless, the performance of mass viral neutralization serology need not be the difficult process which it is usually considered to be. In fact, titration of serum for antibody content can be accomplished with greater ease than screening at one dilution by conventional tube technique. In a recent study 7,500 sera were titrated by neutralization tests with adenovirus within one week. This would normally require 10 weeks of

sustained maximum effort(10). Moreover, prolonged testing periods make it virtually impossible to retain cell cultures with similar susceptibility characteristics since these are subject to change with succeeding generations of cells.

Diagnostic virus serology by MTC methods is made more feasible and relatively inexpensive for even the smallest tissue culture laboratory. The reduction of serum requirements is appropriate for neutralizing antibody studies of children or infants where only small amounts are usually available.

Finally, it is possible that many if not all conventional tissue culture techniques can be applied to the MTC method with its subsequent economy and ease of replication so as to greatly enhance virus research as well as diagnostic efforts.

Summary. A simplified method for virus-tissue culture practices accomplished in microtitration plates reduces time, materials, and effort, yet agrees well with results obtained by conventional tissue culture tube methods. Neutralization tests for poliovirus Type III performed by both methods indicated a high degree of compatibility.

Addendum. Since the completion of this study, disposable MTC plates have been produced by the manufacturer.

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POST PUBLICATION ADDENDUM

Use of plastic disposable microtitration plates has shown some variability in the establishment of cell monolayers. It is believed that this is due to either plastic toxicity from molding processes, or in the non-wettability of the plastic material itself.

In most instances, this problem has been solved by dipping the plates in sterile .5% gelatin solution (temperature approximately 60°C) and allowing excess liquid to drain from the plates.

MJR